

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/12, C07K 13/00 C12N 15/62, C12P 21/08 C12Q 1/68, G01N 33/68		A1	(11) International Publication Number: WO 93/23539 (43) International Publication Date: 25 November 1993 (25.11.93)
(21) International Application Number: PCT/US93/04654 (22) International Filing Date: 13 May 1993 (13.05.93)			(74) Agent: PASTERNAK, Sam; Choate, Hall & Stewart, Exchange Place, 53 State Street, Boston, MA 02109 (US).
(30) Priority data: 07/882,711 13 May 1992 (13.05.92)		US	(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
(71) Applicant: DANA-FARBER CANCER INSTITUTE, INC. [US/US]; 44 Binney Street, Boston, MA 02115 (US).			Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(72) Inventors: KAELIN, William, G., Jr. ; 12 Commonwealth Avenue, #301, Boston, MA 02116 (US). FLEMINGTON, Erik ; 32 Russell Street, Brookline, MA 02146 (US). DeCAPRIO, James, A. ; 5 Putney Road, Wellesley, MA 02181 (US). SELLERS, William ; 93 Risley Road, Brookline, MA 02167 (US). LIVINGSTON, David, M. ; 11 Powell Street, Brookline, MA 02146 (US).			

(54) Title: RETINOBLASTOMA-ASSOCIATED PROTEIN 1 cDNA

(57) Abstract

We have discovered a nuclear protein in normal human cells, "retinoblastoma-associated protein 1" ("RBAP-1") that binds directly to the retinoblastoma protein pocket of the underphosphorylated form of the retinoblastoma protein ("RB") and does not bind to phosphorylated RB or to RB with inactivating mutations. The translated RBAP-1 sequence does not resemble other proteins whose sequences are known, and RBAP-1 does not contain a sequence homologous to the transforming element common to viral proteins that bind to the RB pocket. RBAP-1 and the E2F transcription activity have similar DNA-binding specificities and can bind to at least some of the same proteins, such as RB and E4.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LJ	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TC	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam

RETINOBLASTOMA-ASSOCIATED PROTEIN 1 cDNA

Background of the Invention

5 This invention was made in the course of work supported in part by U.S. Government funds, and the government has certain rights in the invention.

This invention relates to tumor suppressor genes.

The human retinoblastoma gene ("RB-1") is considered to be the prototype of a class of genes, generally known as "tumor suppressor genes", thought to be 10 involved in suppressing neoplastic growth. Mutations in the retinoblastoma gene and dysfunction of its product have been implicated in the pathogenesis of a wide range of human tumors other than retinoblastomas, including bladder, breast, and small cell lung carcinomas, osteosarcomas, and soft tissue sarcomas. Furthermore, in cell populations where both copies of *RB-1* are mutated, 15 introduction of a wild-type copy of the gene can lead to a decrease in the growth rate or in the tumorigenicity of the cells expressing the exogenous gene (Huang *et al.*, 1988, *Science*, Vol. 242, pp. 1563-1566). The retinoblastoma gene product, "RB", is believed to regulate cell growth, although the manner in which it does so is not well understood.

20 Several viral transforming proteins, the adenovirus E1A protein ("E1A"), the simian virus large T antigen ("T"), and the human papilloma virus E7 protein ("E7"), bind specifically to RB. The binding of the viral proteins to RB has been mapped to a region of RB termed the "pocket" (Hu *et al.*, 1990, *EMBO J.*, Vol. 9, pp. 1147-1155; Kaelin *et al.*, 1990, *Mol. Cel. Biol.*, Vol. 10, pp 25 3761-3769; Huang *et al.*, 1990, *EMBO J.*, Vol. 9, pp. 1815-1822). The viral proteins share a short, homologous, colinear, transforming element, having at its core the amino acid sequence LXCXE, that is capable of binding to the RB pocket. A synthetic peptide of this viral element is capable of binding to the RB pocket and when bound blocks the binding of viral proteins to the RB pocket.

30 Analysis of RB throughout the cell cycle has demonstrated that it is phosphorylated and dephosphorylated at specific stages of the cell cycle. RB is non-phosphorylated, or "underphosphorylated", in the G₀ and G₁ phases and becomes phosphorylated at the start of S phase, the G₁/S boundary, and remains phosphorylated throughout S phase, G₂ and early mitosis (Buchkovich *et al.*,

1989, *Cell*, Vol. 58, pp. 1097-1105; Chen *et al.*, 1989, *Cell*, Vol. 58, pp. 1193-1198; DeCaprio *et al.*, 1989, *Cell*, Vol. 58, pp. 1085-1095; Mihara *et al.*, 1989, *Science*, Vol. 246, pp. 1300-1303; Xu *et al.*, 1989, *Oncogene*, Vol. 4, pp. 807-812). In terminally differentiated cells and cells that are induced to terminally differentiate, RB is underphosphorylated (Mihara *et al.*, 1989; Furukawa *et al.*, 1990, *Proc. Natl. Acad. Sci. USA*, Vol. 87, pp. 2770-2774). Interaction of the viral transforming proteins with RB is cell-cycle regulated. For example, T does not bind to the phosphorylated form of RB (Ludlow *et al.* 1989, *Cell*, Vol. 56, pp. 57-65), suggesting that some of the growth suppressor functions of RB may be carried out by the underphosphorylated form of RB. The interaction of the viral transforming proteins with RB at specific stages of the cell cycle further supports the proposal that RB is involved in the pathogenesis of some human cancers.

Summary of the Invention

15 We have discovered a nuclear protein in normal human cells, here termed "retinoblastoma-associated protein 1" ("RBAP-1") that, based on *in vitro* evidence, binds directly to the RB pocket of the underphosphorylated form of RB and does not bind to phosphorylated RB or to RB with inactivating mutations. The direct binding of RBAP-1 to RB suggests that RBAP-1 is involved in the RB 20 signal transduction pathway.

We have fully sequenced a near full length clone of RBAP-1 encoding DNA ("RBAP-1"), and deduced the RBAP-1 amino acid sequence. A search of DNA sequence data bases reveals that *RBAP-1* does not resemble other proteins whose DNA sequences are known. The deduced amino acid sequence of 25 RBAP-1 also reveals that RBAP-1 does not contain a colinear sequence, LXCXE, homologous to the transforming element common to viral proteins that bind to RB, although it appears to bind to the same region of RB as do the viral transforming elements.

Analysis of *RBAP-1* gene expression in cell culture demonstrated that 30 *RBAP-1* is expressed primarily during S phase of the cell cycle. RB becomes phosphorylated at the beginning of S phase and we propose that RBAP-1 carries out a function related to the entry into, or traversal of, S phase by the cells.

Analysis of the DNA-binding properties of RBAP-1 has demonstrated that RBAP-1 binds to a DNA sequence that is also bound by cellular extracts that contain an activity known as E2F. E2F activity was originally described as an E1A-targeted component of the functional transcription complex of the adenovirus promoter and was later shown to be normally complexed to cellular proteins in most cell types. E2F has been functionally defined as a transcription factor that is a DNA-binding protein, and more recently has been shown to be a cellular target of RB (Bandara *et al.*, 1991, *Nature*, Vol. 351, pp. 494-497; Chellappan *et al.*, 1991, *Cell*, Vol 65, pp. 1053-1061; Bagchi *et al.*, 1991, *Cell*, Vol. 65, pp. 1063-1072; Chittenden *et al.*, 1991, *Cell*, Vol. 65, pp. 1073-1082). Also, E2F activity containing extracts have been shown to bind to the RB pocket, and this protein complex can be disrupted by E1A or E7. Interestingly, the RB bound E2F activity can recognize more than one DNA sequence (Chittenden *et al.*, 1991). The binding of more than one DNA sequence by the E2F activity suggests that E2F may be a family of proteins.

In one general aspect the invention features a portion of a normal human nuclear protein that is capable of binding to the RB pocket.

In preferred embodiments the human nuclear protein is RBAP-1, having the sequence shown in Fig. 1. In other preferred embodiments RBAP-1 is synthesized *in vitro* using an RBAP-1 encoding DNA, or is made *in vivo* using an RBAP-1 encoding DNA or using the *RBAP-1* gene. In some embodiments the portion of the human nuclear protein is the RB pocket binding portion of RBAP-1, comprising the nucleotide sequence 1191-1397 as shown in Fig. 1, or may be some other portion, and may be the entire RBAP-1 protein.

In another general aspect, the invention features a RBAP-1 encoding DNA. In preferred embodiments the RBAP-1 encoding DNA includes the nucleotide sequence shown in Fig. 1. In some embodiments the invention features a vector containing a portion of the RBAP-1 encoding DNA and may contain the entire RBAP-1 encoding DNA.

30 In another general aspect the invention features a vector containing a *RBAP-1* gene.

In another general aspect, the invention features a method for diagnosing a condition of tumorigenicity in a subject, including the steps of obtaining a tissue sample from a subject and detecting the presence of non-wildtype *RBAP-1* encoding gene in the sample, or detecting alterations in the expression of 5 wildtype *RBAP-1* encoding gene in the sample. "Alteration of expression" as used herein includes an absence of expression, or a substantially decreased expression, or an overexpression of the gene.

In another general aspect, the invention features a nucleic acid probe 10 complementary to a portion of a *RBAP-1* gene. The complementary nucleic acid probe, as used herein, can be complementary to any portion of a *RBAP-1* gene including sense and anti-sense strands of the gene, and including coding and non-coding sequences.

In another general aspect, the invention features a ligand capable of binding 15 to the *RBAP-1* protein. In preferred embodiments, the ligand can bind to the *RBAP-1* protein or to an *RBAP-1/RB* protein complex. The ligand can be a protein other than *RB*, a fusion protein, a polypeptide, or a small molecule. 20 "Small molecule", as that term is used herein, means a chemical compound, a peptide, an oligonucleotide, having a sequence other than the sequences known to be bound by the E2F activity, or a natural product. Preferably the small molecule is a therapeutically deliverable substance.

In another general aspect, the invention features a ligand that is capable of 25 altering the activation of a gene by *RBAP-1*. The ligand may alter gene activation by *RBAP-1* by decreasing, the affinity of *RBAP-1* for the specific DNA site, or decreasing, *RBAP-1* transactivation of the promoter that is downstream from the DNA binding site.

In another general aspect, the invention features a ligand that is capable of 30 disrupting the interaction of a viral transforming protein and *RB*, while not disrupting the interaction of *RBAP-1* and *RB*.

In another general aspect the invention features methods for assaying for a 35 ligand that is capable of disrupting the interaction of a viral transforming protein and *RB*, while not disrupting the interaction of *RBAP-1* and *RB*. In one aspect the method comprises the steps of: immobilizing *RB* on a solid support;

contacting the ligand and a viral transforming protein with the immobilized RB and separately contacting the ligand and RBAP-1, or a RB binding portion of RBAP-1; determining binding of the viral protein to RB and of RBAP-1 to RB in the presence of the ligand.

5 In another aspect the method comprises the steps of: transforming a first cell with vectors containing a reporter gene having an activatable promoter, and containing DNA encoding RB and RBAP-1 where one of the potential binding partners is fused to a transactivating domain and the other is fused to a site specific DNA binding domain; transforming a second cell with vectors containing 10 a reporter gene having an activatable promoter, and containing DNA encoding RB and one viral transforming protein where one of the potential binding partners is fused to a transactivating domain and the other is fused to a site specific DNA binding domain; culturing the transformed cells in the presence of a ligand and determining the expression of the reporter gene. A preferred "cell" 15 is a cultured eukaryotic cell, such as a yeast, for example *S. cerevisiae*, or a mammalian cell. An "activatable promoter", as used herein, is a promoter having a sequence specific binding site upstream of the transcriptional start site that is activated by the binding of a sequence specific DNA binding domain to the specific site and the proximity of a transactivating domain to the DNA binding 20 domain. Each of these domains is fused to one protein of a pair that can interact to form a protein-protein complex and thus the domains are brought into the proximity required to activate transcription from the gene.

 In another aspect the method comprises the steps of: transforming a mammalian cell expressing a viral transforming protein with vectors containing a 25 reporter gene having an activatable promoter, and containing DNA encoding RB and RBAP-1 where one of the potential binding partners is fused to a transactivating domain and the other is fused to a site specific DNA binding domain; culturing the transformed cell in the presence of a ligand and determining expression of the reporter gene.

30 In another general aspect, the invention features a monoclonal antibody directed to RBAP-1. In preferred embodiments the monoclonal antibody is

directed against a portion of RBAP-1 including the amino acid residues encoded by nucleotides 1191-1655 of RBAP-1 encoding DNA, as shown in Fig 1.

In another general aspect the invention features eukaryotic homologues of RBAP-1. In preferred embodiments that eukaryotic homologues 5 have been cloned using a portion of the RBAP-1 encoding DNA sequence as a probe.

Description of the Preferred Embodiments

Drawings

Fig. 1 is a diagram showing the nearly complete nucleic acid sequence of 10 the RBAP-1 encoding DNA, and the deduced amino acid sequence. A candidate initiator methionine, M, is shown, although the sequence 5' of the corresponding ATG is open. The underlined sequence is the sequence of RBAP-1 that contains 20 the RB pocket binding site.

Cloning and Characterization of RBAP-1

15 The following description, presented by way of example, details the cloning and characterization of RBAP-1. It will be appreciated that the genes of proteins that bind to RBAP-1 or the RBAP-1/RB protein complex can be cloned and characterized in an analogous manner.

The RBAP-1 encoding DNA was cloned from a λ gt11 expression library 20 using radiolabelled RB ("*RB") as a probe, according to cloning techniques generally known in the art (see for example, Singh *et al.*, 1989, *Biotechniques*, Vol. 7, pp. 252-162). *RB was prepared using the pGEX-2TK plasmid which is a modification of the commercially available expression vector pGEX-2T (Pharmacia). pGEX-2T has been modified to encode a GST fusion protein in 25 which a recognition sequence for the catalytic subunit of cAMP dependent protein kinase from heart muscle was interposed between the GST leader polypeptide and the polypeptide encoded by the inserted cDNA. Briefly, the library was plated at approximately 40,000 pfu/150 mm plate on 30 plates (pfu = plaque forming units). The expression of β -galactosidase fusion proteins was 30 induced using IPTG impregnated nitrocellulose. The nitrocellulose was probed with *RB by incubating the nitrocellulose in a buffered solution containing *RB, and the unbound proteins were washed off. The plaques corresponding to the

fusion proteins bound by *RB were picked and purified using further rounds of hybridization as is standard in the art. The DNA from the pure plaques was prepared and the sequence representing the RB-binding fusion protein was subcloned into pBKSTTM (Stratagene) for sequencing. DNA sequencing was 5 performed using a SequenaseTM 2.0 kit (available from United States Biochemical Corp.) according to a protocol provided by the manufacturer.

Analysis of the DNA sequence demonstrated that 4 of the clones contained overlapping DNA sequence and were derived from a common mRNA. 10 Additional clones were obtained by screening another library and rescreening the original library with one of the above clones. The 2465 bp sequence of *RBAP-1* was deduced from examination of multiple clones; the sequence shown in Fig 1 is nearly the full length sequence of *RBAP-1* encoding DNA, but may be missing about 500 bp from the 5' end, as determined by Northern Blot analysis.

The binding of the fusion proteins that comprise *RBAP-1* to RB was 15 characterized *in vitro*. Briefly, the purified λ phage of each fusion protein was plated on a separate plate, and the expression of the β-galactosidase fusion proteins was induced using IPTG impregnated nitrocellulose. The proteins on the nitrocellulose were renatured (see Vinson *et al.*, 1988, *Genes & Dev.*, Vol. 2, pp. 801-806) and probed with *RB by incubating the nitrocellulose in a buffered 20 solution containing *RB. The proteins that were bound by *RB were visualized by autoradiography. The results of the autoradiograph demonstrated that all 4 fusion proteins of *RBAP-1* were capable of binding directly to RB.

The ability of these proteins to bind to the RB pocket was determined by probing the nitrocellulose with a radiolabelled non-binding mutant of RB, and 25 with *RB in the presence of a synthetic peptide homologous to the E7, E1A, T, viral transforming element. The results showed that the fusion proteins bound directly to the RB pocket and that the binding of these fusion proteins to RB could be significantly reduced or blocked by the viral transforming element.

Nucleotides 1191-1655 of the *RBAP-1* encoding DNA, as shown in Fig. 1, 30 were subcloned into pGEX-2T (see Kaelin *et al.*, 1991), to create a glutathione S-transferase fusion protein ("GST-RBAP-1"), and used to determine whether RBAP-1 could bind to the phosphorylated or the underphosphorylated form of

RB synthesized *in vivo*. Briefly, GST-RBAP-1 was purified from *E. coli* and bound to glutathione-Sepharose™, a glutathione-linked cellulose gel (Pharmacia). RB was prepared from asynchronously growing cells and incubated with the Sepharose™ bound GST-RBAP-1. After washing, bound proteins were eluted 5 from the Sepharose™ and immunoblotted with a monoclonal antibody against RB (monoclonal 245 available from Pharmigen). The results demonstrated that RBAP-1 specifically binds to the underphosphorylated form of RB.

The expression of the *RBAP-1* gene was investigated using Northern analysis. The Northern analysis was performed using a RBAP-1 encoding DNA 10 probe and total RNA obtained from peripheral blood T lymphocytes that were resting (G₀ cells), blocked at the G₁/S boundary, and synchronously growing. The results demonstrated that *RBAP-1* mRNA accumulates when the cells are blocked at the G₁/S boundary and falls after S phase.

The physical properties of RBAP-1 were examined using techniques that 15 are well known in the art, and RBAP-1 was determined to have the same DNA-binding sequence specificity as E2F. Briefly, RBAP-1 co-purifies with E2F activity on DNA affinity columns (see, Means *et al.*, 1992, *Mol. Cel. Biol.*, Vol. 12, pp. 1054-1063). RBAP-1 immunoprecipitated from cell extracts using the monoclonal antibody against RBAP-1, described herein, and bacterially 20 produced RBAP-1 were shown to contain E2F activity by non-denaturing polyacrylamide gel shift analysis using the E2F DNA-binding site for E2F (see, Shirodkar *et al.*, 1992, *Cell*, Vol. 68, pp. 157-166). Additionally, RBAP-1 binds to the adenovirus E4 protein, a protein that is known to specifically bind to E2F, as was demonstrated by the binding of RBAP-1 to a GST-E4 fusion protein 25 using the method described above (Kaelin *et al.*, 1991).

The role of RBAP-1 *in vivo*

Without being limited thereby, we here propose a theory of a role of the RBAP-1 protein *in vivo*. We demonstrated that the *RBAP-1* gene is expressed just prior to the point in the cell cycle that RB is phosphorylated, and that 30 RBAP-1 binds specifically to the pocket of the underphosphorylated form of RB. Moreover, the RB pocket region is frequently mutated in human tumors and the

underphosphorylated form of RB is thought to have tumor suppressing effects in that this form is believed to inhibit the progression of the cell cycle.

We propose two alternative models for the significance of RBAP-1 binding to RB. In one model, if RBAP-1 is present before the onset of RB phosphorylation, the binding of RBAP-1 to RB can lead to RB phosphorylation. In this manner RBAP-1 would act "upstream" of RB in a signal transduction pathway and bring about the phosphorylation of RB. This model is consistent with the observation that loss of function RB mutants are hypophosphorylated *in vivo*, suggesting that cellular ligands of RB must bind to the RB pocket before phosphorylation can occur. Alternatively, RBAP-1 may be a "downstream" target of RB. In this model RBAP-1 binds to dephosphorylated RB generated near the end of M phase or is bound by newly synthesized RB that has not undergone post-translational modification.

Use

The invention provides for identification of ligands that bind to RBAP-1 or the RBAP-1/RB complex, identification of ligands that disrupt the binding of RB to a viral transforming protein, or the viral transforming element, and do not effect the binding of RB to RBAP-1, production of monoclonal antibodies directed to RBAP-1 or any peptide of RBAP-1, and detection of non-wild-type RBAP-1 genes or detection of alteration in the expression of wild-type *RBAP-1* genes.

Identification of ligands that bind to RBAP-1 or RBAP-1/RB.

The RBAP-1 protein can be used to identify ligands that bind to or interact with RBAP-1 or with the RB/RBAP-1 complex. The identification of ligands that bind to RBAP-1 or the RBAP-1/RB complex can be approached using the same method by which RBAP-1 was cloned. For instance, labelled RBAP-1 or a complex of labelled RBAP-1/RB can be used as probes for expression libraries of fusion proteins, and the DNA encoding the protein that binds to either RBAP-1 or the RBAP-1/RB complex can be cloned generally as described above.

Radioactive labelling is a preferred method for convenient labelling of proteins.

RBAP-1 or a complex of RBAP-1/RB can be used to screen a peptide library. The screening of a peptide library can be done using techniques

generally known in the art (see for example Scott *et al.*, 1990, *Science*, Vol. 249, pp. 386-390; Devlin *et al.*, 1990, *Science*, Vol. 249, pp. 404-406; Lam *et al.*, 1992, *Nature*, Vol. 354, pp. 82-84). Briefly, RBAP-1 can be linked to a reporter gene, such as alkaline phosphatase ("AP") by cloning an in-frame fusion of RBAP-1 and AP ("AP/RBAP-1"), and used to screen a library of peptides linked to beads. The binding of AP/RBAP-1 to beads can be determined by staining and the amino acid sequence of the peptide on the bead determined by sequencing with a microsequencer (Lam *et al.*). In another approach, RBAP-1 can be attached to a solid support, such as a petri dish, and an epitope library, a peptide library inserted into a coat protein of filamentous phage such that the peptide is on the surface of the phage capsule, can be passed over the RBAP-1. Successive rounds of binding to RBAP-1 and propagating the phage that bind to RBAP-1 allows the purification of the individual phage clones (Scott *et al.*; Devlin *et al.*). The sequence of the peptide that binds to RBAP-1 can be determined by sequencing the DNA.

An *in vitro* assay for ligands, especially small molecules, that interact with RBAP-1 and alter its binding to DNA can be established, for example, by immobilizing RBAP-1 on a solid support, such as a microtiter tray well. The immobilized RBAP-1 can be incubated with a mixture of a ligand and a labelled DNA fragment, containing a sequence bound by the E2F activity. After incubation, the well can be washed to remove unbound species and the amount of label remaining in the well can be measured. A ligand that binds to RBAP-1 and disrupts the binding of RBAP-1 to the labelled DNA fragment can be detected by an absence of label remaining in the well.

Alternatively, an *in vivo* assay for ligands that bind to RBAP-1 and alter the activation of a gene that is transactivated by RBAP-1 can be established. For example this assay can be accomplished by transforming a cell, such as the yeast cell *S. cerevisiae*, with a reporter gene, such as β -galactosidase, under the control of an activatable promoter that has a sequence bound by the E2F activity upstream of the promoter. The cell is also transformed with a plasmid encoding RBAP-1. These cells can be grown in the presence of the chromogenic substrate X-gal, and the cells will produce a blue pigment if the β -galactosidase reporter

gene is transactivated by RBAP-1 and transcribed. Cells can be cultured in the presence of different ligands and the ability of the ligand to disrupt the transactivation of the reporter gene can be measured by assaying for the disappearance of the blue color from cell colonies. Ligands that alter the 5 activation of a gene by RBAP-1, either by decreasing the binding of RBAP-1 to the DNA or by decreasing the transactivation of the gene by RBAP-1 may be useful for therapeutic treatment of individuals that are lacking functional RB.

Identification of ligands that disrupt RB binding to viral transforming proteins without disrupting RB binding to RBAP-1.

10 RBAP-1 can be used to identify ligands that bind to RB and disrupt the binding of the viral transforming proteins to RB without affecting the binding of RBAP-1 to RB. The following methods for the identification of ligands are described for purposes of example only, and as will be appreciated methods within the invention may differ in particulars from those described.

15 An *in vitro* assay for ligands that disrupt the binding of RB and a viral RB binding protein, such as E7, E1A or T, can be established by immobilizing RB, RBAP-1 or a viral RB binding protein on a solid support, such as in a microtiter tray well. For example, RB can be immobilized on the solid support, and a mixture of a ligand and either labelled RBAP-1 ("*RBAP-1") or labelled E7 20 ("*E7") can be added to the wells of the microtiter plate. After incubation the wells can be washed to remove unbound species and the amount of label remaining in the well determined. A ligand that disrupts binding to RB can be detected by an absence of label remaining in the well. In particular, a ligand that specifically disrupts the binding of RB to E7 would be demonstrated by a lack of 25 label remaining in the well where RB, *E7 and the ligand had been incubated together, and the presence of label in the well where RB, *RBAP-1, and the same ligand had been incubated. Radioactive labelling of the proteins is a preferred method for convenient labelling of proteins.

30 An *in vivo* assay for ligands can be established, for example, by using the yeast *S. cerevisiae* that contains a reporter gene, such as β -galactosidase, under the control of an activatable promoter, such as a promoter with multiple GAL4 binding sites. *S. cerevisiae* can be transformed with plasmids encoding chimeric

proteins in which the DNA-binding region of GAL4 can be fused to RB ("GAL4-RB") and the transactivating region of VP16 can be fused to E7 ("VP16-E7") (see for example, Fields *et al.*, 1989, *Nature*, Vol. 340, pp. 245-246; Dang *et al.*, 1991, *Mol. Cell. Biol.*, Vol. 11, pp. 954-962). These cells can be grown in
5 the presence of the chromogenic substrate X-gal, and the cells produce a blue pigment if GAL4-RB fusion binds to the VP16-E7 fusion protein. The cells can be cultured in replicate in the presence of different ligands and the ability of the ligand to disrupt the binding of RB and E7 can be evidenced by an absence of blue pigment produced by the cells. In order to confirm a specific interaction
10 between E7 and Rb, the ligand can be tested for its ability to disrupt the binding of RBAP-1 to RB using a VP16-RBAP-1 fusion protein in place of the VP16-E7 fusion protein in a similar assay.

An *in vivo* assay for ligands that disrupt the binding of RB and a viral transforming protein can alternatively be established in a mammalian cell in an
15 analogous manner. For example a cervical carcinoma cell that expresses E7, such as HeLa cells, can be transformed with DNA-binding and transactivating fusion proteins of RB and RBAP-1, and a reporter gene downstream of an activatable promoter. These cells can be grown in the presence of different ligands in order to find a ligand that is capable of restoring the binding between
20 RB and RBAP-1.

Ligands that appear to disrupt the binding of RB to the viral transforming element without disrupting the binding of RB to RBAP-1, can be assayed for the specificity of this disruption by determining their capacity to interfere with the binding of an unrelated pair of binding proteins. If the ligand is unable to
25 disrupt the binding of other binding proteins then it can be concluded that the ligand interacts specifically with either RB or the viral transforming element to disrupt their binding.

A ligand that selectively disrupts RB binding to the viral transforming element may do so by binding to RB in such a way as to prevent the binding of
30 the transforming element without disrupting the normal RB/RBAP-1 interaction, or may bind to the transforming element with a higher affinity than the affinity of RB and the transforming element. In either case such a ligand can be used in

treatment of individuals suffering from a pathologic disease state, such as cervical carcinoma or a malignancy in which the RB signal transduction pathway has been disrupted.

5 Deletions of the RBAP-1 encoding DNA to define functional portions of the protein.

The RB pocket binding region of RBAP-1 can be further defined by constructing deletions of the RBAP-1 encoding DNA and determining binding of the proteins encoded by these deletion mutants to the RB pocket. Deletion mutations of *RBAP-1* can be constructed from knowledge of the *RBAP-1* sequence using techniques well known in the art. For example, a polymerase chain reaction technique can be used to construct a subclone of a specific portion of the DNA; or a series of deletion constructs, such as 3' deletions, can be constructed by cutting the DNA at a convenient restriction endonuclease site upstream of the stop codon and digesting the DNA with an exonuclease to 10 produce a series of deletions in the 3' end of the DNA. Proteins encoded by deletion mutants of *RBAP-1* can be assayed for their ability to specifically bind to 15 the RB pocket as described above.

Monoclonal antibodies against RBAP-1.

Monoclonal antibodies were raised against a peptide of RBAP-1 using 20 techniques generally known in the art (Harlow *et al.*, 1988, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 6). As will be understood, a monoclonal antibody against any portion of RBAP-1 can be produced using the techniques described below, or any of the techniques described by Harlow *et al.*

25 Briefly, the GST-RBAP-1 fusion protein described above, that is composed of nucleotides 1191-1655 of Fig. 1 fused to glutathione S-transferase, was overexpressed in *E. coli* and isolated. The fusion protein was suspended in complete Freund's adjuvant and injected intraperitoneally into mice. Each mouse was boosted with the isolated fusion protein in incomplete Freund's adjuvant by 30 another intraperitoneal injection approximately two weeks later, and serum was collected from the mouse by tail bleed an additional 10 or more days later. The serum was tested for antibodies against RBAP-1, and subsequent rounds of

boosting and bleeding were done as necessary. Serum samples were checked for specific recognition of *RBAP-1* by immunoprecipitation of radiolabelled *RBAP-1* and the mice which had produced the best response were prepared for hybridoma fusion. The final booster injection was given 3 weeks after the latest boost, and

5 about 3 days prior to hybridoma fusion, the booster was delivered both as an intravenous injection and an intraperitoneal injection. The spleen was removed from the immunized mice and the cells were separated. The spleen cells were fused to myeloma cells with polyethylene glycol, and the fused cells were aliquoted into wells of a microtiter plate. The cells were grown in selective

10 medium to select for the growth of hybridoma cells only. The wells containing colonies of hybridomas were screened by removing a portion of the cell culture supernatant and detecting the secretion of antibodies by antibody capture on permeabilized cells or in solution. The specific hybridoma colony that secretes antibody was cloned by limiting dilution and expanded by growing in

15 successively larger containers.

Detection of non-wild-type *RBAP-1* or alterations in expression of wild-type *RBAP-1*.

The detection of alterations of expression of wild-type *RBAP-1* or the presence of non-wild-type *RBAP-1* in a tissue sample from a subject, using

20 techniques well known in the art, can provide for early diagnosis of a neoplasm. The following methods are presented for purposes of example only, the methods employed can differ from the described methods and remain within the spirit of the invention.

Alterations in the level of *RBAP-1* expression can be detected by a well

25 known technique such as Northern blotting of the *RBAP-1* mRNA.

Mutations in the *RBAP-1* gene, including point mutations and specific deletions or insertions of the coding sequence, the 5' untranslated region and the

30 3' untranslated region, can be detected by cloning and sequencing the *RBAP-1* allele present in the sample taken from the subject. If desired, the *RBAP-1* mRNA can be sequenced directly, or the polymerase chain reaction technique ("PCR") can be used to amplify *RBAP-1* or its mRNA to produce encoding DNA ("cDNA") and the resultant cDNA can be sequenced. PCR can also be used to

selectively amplify a region of the *RBAP-1* allele; this can be especially useful to identify mutations at the splice-donor sites and in the 3' and 5' untranslated regions.

Mutations in the *RBAP-1* gene can alternatively be detected using single strand conformation polymorphisms (Orita *et al.*, 1989, *Proc. Natl. Acad. Sci., USA*, Vol. 86, pp. 2766-2770). This technique detects deletions and is sensitive enough to detect nucleotide substitutions. For the analysis, *RBAP-1* can be cloned from a sample taken from the subject, or the genomic DNA can be prepared from the sample and either amplified using the polymerase chain reaction technique ("PCR") or directly digested with a restriction endonuclease. If the DNA sample is cloned or prepared by PCR then the sample can be radiolabelled, denatured, and subjected to neutral polyacrylamide gel electrophoresis. The gel can be dried and exposed to film to determine any differences in mobility between the sample from the patient and the wild-type *RBAP-1* control sample. If the DNA sample is prepared by digestion of genomic DNA, it is denatured, subjected to neutral polyacrylamide gel electrophoresis, and the single-stranded DNA's are transferred to nitrocellulose or nylon membrane. The transferred DNA's are probed with radiolabelled *RBAP-1* and any differences in mobility between the DNA from the sample and the wild-type *RBAP-1* control can be visualized by an autoradiographic exposure of the DNA's.

Mutations in the *RBAP-1* gene can also be detected using a nucleic acid probe that is complementary to a portion of *RBAP-1*. This technique is traditionally used to detect point mutations, and one can use a riboprobe (sense or antisense) which is complementary to the wild-type *RBAP-1* gene sequence to detect point mutations in the coding DNA. The riboprobe is first annealed to either mRNA or DNA isolated from the tissue sample, then cleaved with ribonuclease to specifically cleave the riboprobe at mismatches between it and the sample. The cleaved products are separated by gel electrophoresis, and mismatches are detected as segments of the riboprobe smaller than the full length riboprobe. The point mutations can also be detected using a DNA probe. Mutations in the *RBAP-1* gene that have previously been identified can be

detected using allele-specific probes containing a gene sequence corresponding to that mutation. Presence of a specific mutation is confirmed when an allele-specific probe hybridizes with *RBAP-1* sequences from the sample.

Cloning of the genomic RBAP-1 DNA

5 The *RBAP-1* gene can be cloned, for example, by first screening Southern blots of restriction endonuclease digests of genomic DNA from normal peripheral blood lymphocytes with labelled *RBAP-1* encoding DNA to determine the size of the *RBAP-1* gene and determine an appropriate cosmid library with which to pursue the cloning of the gene. The cosmid library can then be screened using

10 conventional techniques with labelled *RBAP-1* encoding DNA and the *RBAP-1* gene can be subcloned into an appropriate plasmid vector, such as pBluescript™ which is a useful cloning vector that contains a polylinker that is flanked by standard primer sequences (Vector), and sequenced.

Cloning of eukaryotic homologues to RBAP-1

15 The *RBAP-1* encoding DNA ("cDNA") can be used to select probes to clone the cDNA or genomic DNA that encodes the *RBAP-1* homologue in other eukaryotic species. A "homologue", as that term is used herein, means a protein in another eukaryotic species that has the same functional properties as the *RBAP-1* protein in humans. Techniques for cloning homologues to a known gene are generally known in the art. For example, a Southern blot of DNA from a desired eukaryote can be screened at low stringency using a labelled portion of *RBAP-1* probe or a labelled oligodeoxynucleotide, that was chosen based upon the *RBAP-1* sequence, as a probe, and the *RBAP-1* homologue can be cloned using an appropriate DNA library from the eukaryote (see for example,

20 25 Sambrook *et al.*, 1989, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press). In another technique the sequence of the *RBAP-1* cDNA can be used to design degenerate oligodeoxynucleotide primers, and a polymerase chain reaction can be conducted using the degenerate primers and DNA from a desired eukaryote (see for example, Hanks *et al.*, 1987, *Proc.*

30 *Natl. Acad. Sci., USA*, Vol. 84, pp. 388-392; Lee *et al.*, 1988, *Science*, Vol. 239, pp. 1288-1291)

Other embodiments are within the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Kaelin Jr., William G.
Flemington, Erik
Sellers, William
DeCaprio, James A.
Livingston, David M.

(ii) TITLE OF INVENTION: Retinoblastoma-Associated Protein 1 cDNA

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Choate, Hall & Stewart
(B) STREET: Exchange Place, 53 State Street
(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: U.S.A.
(F) ZIP: 02109

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US
(B) FILING DATE: 13-MAY-1992
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Kaplan, Warren
(B) REGISTRATION NUMBER: 34,199
(C) REFERENCE/DOCKET NUMBER: DFCI#236PCT

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (617) 227-5020
(B) TELEFAX: (617) 227-7566
(C) TELEX: 289374

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2456 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGGATCGAGC CCTCGCCGAG GCCTGCCGCC ATGGGCCCGC GCCGCCGCCG CCGCCTGTCA

CCCGGGCCGC	GCAGGGCCGTG	AGCGTCATGG	CCTTGGCCGG	GGCCCGTGCG	GGCGGGCCAT	120
GCAGCGCCGGC	GCTGGAGGCC	CTGCTCGGGG	CCGGCGCGCT	GCGGCTGCTC	GACTCCTCGC	180
AGATCGTCAT	CATCTCCGCC	GCAGCAGGACG	CCAGCGCCCC	GCCGGCTCCC	ACCGGCCCCG	240
CGGCGCCGC	CGCCGGCCCC	TGCGACCCCTG	ACCTGCTGCT	CTTCGCCACA	CCGCAGGCGC	300
CCCAGCCAC	ACCCAGTGC	CCGCGGCCCG	CGCTCGGCCG	CCCGCCGGTG	AAGCGGAGGC	360
TGGACCTGGA	AACTGACCAT	CAGTACCTGG	CCGAGAGCAG	TGGGCCAGCT	CGGGGCAGAG	420
GCCGCATCC	AGGAAAAGGT	GTGAAATCCC	CGGGGGAGAA	GTCACGCTAT	GAGACCTCAC	480
TGAATCTGAC	CACCAAGCGC	TTCTGGAGC	TGCTGAGCCA	CTCGGCTGAC	GGTGTGTCG	540
ACCTGAAC	TG	GGCTGCCGAG	GTGCTGAAGG	TGCAGAAGCG	GCGCATCTAT	600
ACGTCCITGA	GGGCATCCAG	CTCATGGCCA	AGAAGTCCAA	GAACCACATC	CAGTGGCTGG	660
GCAGCCACAC	CACAGTGGGC	GTCGGCGGAC	GGCTTGAGGG	GTTGACCCAG	GACCTCCGAC	720
AGCTGCAGGA	GAGCGAGCAG	CAGCTGGACC	ACCTGATGAA	TATCTGTACT	ACGCAGCTGC	780
GCCTGCTCTC	CGAGGACACT	GACAGCCAGC	GCCTGGCTTA	CGTGACGTGT	CAGGACCTTC	840
GTAGCATTGC	AGACCCCTGCA	GAGCAGATGG	TTATGGTGAT	CAAAGCCCT	CCTGAGACCC	900
AGCTCCAAGC	CGTGGACTCT	TCGGAGAACT	TTCAAGATCTC	CCTTAAGAGC	AAACAAGGCC	960
CGATCGATGT	TTTCTCTGTC	CCTGAGGAGA	CCGTAGGTGG	GATCAGCCCT	GGGAAGACCC	1020
CATCCCAGGA	GGTCACCTCT	GAGGAGGAGA	ACAGGGCCAC	TGACTCTGCC	ACCATAGTGT	1080
CACCAACCAC	ATCATCTCCC	CCCTCATCCC	TCACCAACAGA	TCCCAGCCAG	TCTCTACTCA	1140
GCCTGGAGCA	AGAACCGCTG	TTGTCGGGGA	TGGGCAGCCT	GCAGGGCTCCC	GTGGACGAGG	1200
ACCGCCTGTC	CCCGCTGGTG	GGGGCCGACT	CGCTCTGGGA	GCATGTGCGG	GAGGACTTCT	1260
CCGGCCTCCT	CCCTGAGGAG	TTCATCAGCC	TTTCCCCACC	CCACGAGGCC	CTCGACTTAC	1320
ACTTCGGCCT	CGAGGAGGGC	GAGGGCATCA	GAGACCTCTT	CGACTGTGAC	TTTGGGGACC	1380
TCACCCCCCT	GGATTTCTGA	CAGGGCTGG	AGGGACCAGG	GTTTCCAGAG	ATGCTCACCT	1440
TGTCTCTGCA	GCCCTGGAGC	CCCCTGTCCC	TGGCGTCTCT	CCCAGCCTGT	TTGGAAACAT	1500
TTAATTTATA	CCCCCTCTCCT	CTGCTCCAG	AAGCTCTAG	CTCTGGGTC	TGGCTACCGC	1560
TAGGAGGCTG	AGCAAGCCAG	GAAGGGAAAGG	AGTCTGTGTG	GTGTGTATGT	GCATGCAGCC	1620
TACACCCACA	CGTGTGTACC	GGGGGTGAAT	GTGTGTGAGC	ATGTGTGTGT	GCATGTACCG	1680
GGGAATGAAG	GTGAACATAC	ACCTCTGTGT	GTGCACTGCA	GACACGCC	AGTGTGTCCA	1740
CATGTGTGTG	CATGAGTCCA	TGTGTGCGCG	TGGGGGGCT	CTAACTGCAC	TTTCGGCCCT	1800
TTTGTCTGG	GGGTCCACAA	GGCCCAAGGGC	AGTGCCTGCT	CCCAGAATCT	GGTGCTCTGA	1860
CCAGGCCAGG	TGGGGAGGCT	TTGGCTGGCT	GGGCGTGTAG	GACGGTGAGA	GCACTCTGT	1920
CCTAAAGGTT	TTTCTGATT	GAAGCTTAA	TGGAGCGTTA	TTTATTTATC	GAGGCCTCTT	1980
TGGTGAGCCT	GGGGAATCAG	CAAAGGGGAG	GAGGGGTGTG	GGGTTGATAC	CCCAACTCCC	2040
TCTACCCCTG	AGCAAGGGCA	GGGGTCCCTG	AGCTGTTCTT	CTGCCCCATA	CTGAAGGAAC	2100
TGAGGCCCTGG	GTGATTTATT	TATTGGAAA	GTGAGGGAGG	GAGACAGACT	GAATGACAGC	2160
CATGGGTGGT	CAGATGGTGG	GGTGGGCCCT	CTCCAGGGGG	CCAGTTCAAG	GCCCCAGCTG	2220
CCCCCCAGGA	TGGATATGAG	ATGGGAGAGG	TGAGTGGGGG	ACCTTCACTG	ATGTGGGCAG	2280
GAGGGGTGGT	GAAGGCCTCC	CCCAGCCCAAG	ACCTGTGGT	CCCTCTGCA	GTGTCTGAAG	2340
CGCCTGCCTC	CCCACTGCTC	TGCCCCACCC	TCCAATCTGC	ACTTGTGATT	GCTTCCTAAC	2400
AGCTCTGTT	CCTCCTGCTT	TGGTTTAAT	AAATATTTG	ATGACGTTAA	AAAAAA	2456

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 437 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Ala	Leu	Ala	Gly	Ala	Pro	Ala	Gly	Gly	Pro	Cys	Ala	Pro	Ala	Leu
1		5				10							15		
Glu	Ala	Leu	Leu	Gly	Ala	Gly	Ala	Leu	Arg	Leu	Leu	Asp	Ser	Ser	Gln
		20				25							30		
Ile	Val	Ile	Ile	Ser	Ala	Ala	Gln	Asp	Ala	Ser	Ala	Pro	Pro	Ala	Pro
		35				40						45			
Thr	Gly	Pro	Ala	Ala	Pro	Ala	Ala	Gly	Pro	Cys	Asp	Pro	Asp	Leu	Leu
		50				55					60				
Leu	Phe	Ala	Thr	Pro	Gln	Ala	Pro	Arg	Pro	Thr	Pro	Ser	Ala	Pro	Arg
		65			70			75			80				
Pro	Ala	Leu	Gly	Arg	Pro	Pro	Val	Lys	Arg	Arg	Leu	Asp	Leu	Glu	Thr
		85			90			95							
Asp	His	Gln	Tyr	Leu	Ala	Glu	Ser	Ser	Gly	Pro	Ala	Arg	Gly		
		100			105			110							
Arg	His	Pro	Gly	Lys	Gly	Val	Lys	Ser	Pro	Gly	Glu	Lys	Ser	Arg	Tyr
		115			120			125							
Glu	Thr	Ser	Leu	Asn	Leu	Thr	Thr	Lys	Arg	Phe	Leu	Glu	Leu	Leu	Ser
		130			135			140							
His	Ser	Ala	Gly	Asp	Val	Val	Asp	Leu	Asn	Trp	Ala	Ala	Glu	Val	Leu
		145			150			155				160			
Lys	Val	Gln	Lys	Arg	Arg	Ile	Tyr	Asp	Ile	Thr	Asn	Val	Leu	Glu	Gly
		165			170			175							
Ile	Gln	Leu	Ile	Ala	Lys	Lys	Ser	Lys	Asn	His	Ile	Gln	Trp	Leu	Gly
		180			185			190							
Ser	His	Thr	Thr	Val	Gly	Val	Gly	Gly	Arg	Leu	Glu	Gly	Leu	Thr	Gln
		195			200			205							
Asp	Leu	Arg	Gln	Leu	Gln	Glu	Ser	Glu	Gln	Gln	Leu	Asp	His	Leu	Met
		210			215			220							
Asn	Ile	Cys	Thr	Thr	Gln	Leu	Arg	Leu	Leu	Ser	Glu	Asp	Thr	Asp	Ser
		225			230			235			240				
Gln	Arg	Leu	Ala	Tyr	Val	Thr	Cys	Gln	Asp	Leu	Arg	Ser	Ile	Ala	Asp
		245			250			255							
Pro	Ala	Glu	Gln	Met	Val	Met	Val	Ile	Lys	Ala	Pro	Pro	Glu	Thr	Gln
		260			265			270							
Leu	Gln	Ala	Val	Asp	Ser	Ser	Glu	Asn	Phe	Gln	Ile	Ser	Leu	Lys	Ser
		275			280			285							
Lys	Gln	Gly	Pro	Ile	Asp	Val	Phe	Leu	Cys	Pro	Glu	Glu	Thr	Val	Gly
		290			295			300							
Gly	Ile	Ser	Pro	Gly	Lys	Thr	Pro	Ser	Gln	Glu	Val	Thr	Ser	Glu	Glu
		305			310			315			320				
Glu	Asn	Arg	Ala	Thr	Asp	Ser	Ala	Thr	Ile	Val	Ser	Pro	Pro	Pro	Ser

325 330 335
Ser Pro Pro S r Ser Leu Thr Thr Asp Pro Ser Gln Ser Leu Leu Ser
340 345 350
Leu Glu Gln Glu Pro Leu Leu Ser Arg Met Gly Ser Leu Arg Ala Pro
355 360 365
Val Asp Glu Asp Arg Leu Ser Pro Leu Val Ala Ala Asp Ser Leu Leu
370 375 380
Glu His Val Arg Glu Asp Phe Ser Gly Leu Leu Pro Glu Glu Phe Ile
385 390 395 400
Ser Leu Ser Pro Pro His Glu Ala Leu Asp Tyr His Phe Gly Leu Glu
405 410 415
Glu Gly Glu GIy Ile Arg Asp Leu Phe Asp Cys Asp Phe Gly Asp Leu
420 425 430
Thr Pro Leu Asp Phe
435

Claims

1. A RB pocket binding portion of RBAP-1.
2. The RB pocket binding portion of RBAP-1 in claim 1, having an amino acid sequence including a portion of the amino acid sequence shown in SEQ. ID NO. 1.
3. The RB pocket binding portion of RBAP-1 of claim 2, wherein the said RB pocket binding portion comprises the amino acids from 369-437 as shown in SEQ. ID NO. 1.
4. A substantially purified human protein RBAP-1 encoded by a nucleotide sequence, a portion of which nucleotide sequence is shown in Seq. ID No. 1.
5. The substantially purified human protein RBAP-1 of claim 4 wherein said protein RBAP-1 is capable of binding to protein RB.
6. The substantially purified human protein RBAP-1 of claim 4 wherein said protein RBAP-1 is capable of binding directly to an RB pocket region of protein RB.
7. The substantially purified human protein RBAP-1 of claim 6 wherein said protein RBAP-1 is further capable of binding to an underphosphorylated form of protein RB.
8. The substantially purified human protein RBAP-1 of claim 7 wherein said protein RBAP-1 is further incapable of binding to a phosphorylated form of protein RB.
9. The substantially purified human protein RBAP-1 of claim 4 wherein said protein RBAP-1 is capable of binding to an adenovirus E4 protein.
10. The substantially purified human protein RBAP-1 of claim 4, capable of binding to a pocket region of RB protein, said protein lacking a pentapeptide amino acid sequence having amino acids leucine, cysteine, and glutamic, acid in a first, third, and fifth position respectively.
11. The substantially purified human protein RBAP-1 of claim 10 wherein said protein RBAP-1 is further capable of binding to an underphosphorylated form of protein RB.

12. The substantially purified human protein RBAP-1 of claim 11 wherein said protein RBAP-1 is further incapable of binding to a phosphorylated form of RB.

13. The substantially purified human protein RBAP-1 of claim 4, comprising a peptide fragment capable of binding to a pocket domain of RB protein, said peptide fragment being encoded by nucleotides 1191-1397 of Seq. ID No. 1.

14. The substantially purified human protein RBAP-1 of claim 13, wherein said peptide fragment is encoded by a portion of the nucleotides 1191-1397 of Seq. ID No. 1.

15. A substantially purified eukaryotic protein that binds to protein RB having amino acid homology to protein RBAP-1, a portion of which amino acid sequence of said RBAP-1 protein is shown in Seq. ID No. 2.

16. The eukaryotic protein having homology to RBAP-1 in claim 15, wherein said protein was cloned using a probe derived from the RBAP-1 nucleotide sequence, a portion of which nucleotide sequence is shown in Seq. ID No. 1.

17. An RBAP-1 encoding DNA having a nucleotide sequence including the sequence shown in SEQ. ID No. 1.

18. A vector containing the RBAP-1 encoding DNA of claim 16.

19. A vector containing a RBAP-1 gene.

20. A nucleic acid probe complementary to a RBAP-1 gene.

21. A method for diagnosing a condition of tumorigenicity in a subject, comprising the steps of obtaining a tissue sample from the subject and detecting the presence of a non wild-type RBAP-1 encoding gene in the sample.

22. A method for diagnosing a condition of tumorigenicity in a subject, comprising the steps of obtaining a tissue sample from the subject and detecting an alteration in the expression of a wild-type RBAP-1 encoding gene in the sample.

23. A ligand capable of binding to the RBAP-1 protein.

24. A ligand capable of binding to the RB/RBAP-1 protein complex.

25. The ligand of claims 23 or 24 wherein said ligand is a protein.

26. The ligand of claims 23 or 24 wherein said ligand is a fusion protein.

27. The ligand of claims 23 or 24 wherein said ligand is a polypeptide.
28. The ligand of claims 23 or 24 wherein said ligand is a small molecule.
29. The ligand of claim 23 wherein said ligand is capable of decreasing RBAP-1 transactivation of a gene.
30. The ligand of claim 23 wherein said ligand is capable of decreasing the binding of RBAP-1 to its specific DNA binding site.
31. A ligand capable of disrupting the binding of RB and a viral transforming protein wherein said ligand does not disrupt the binding of the RBAP-1 protein and RB.
32. A method for assaying for a ligand that is capable of disrupting the interaction of a viral transforming protein and RB, while not disrupting the interaction of RBAP-1 and RB comprising the steps of,
 - immobilizing RB on a solid support,
 - contacting RB with one viral transforming protein in the presence of said ligand,
 - separately contacting RB with RBAP-1 in the presence of said ligand,
 - determining binding of the viral transforming protein to RB and binding of RBAP-1 to RB in the presence of said ligand.
33. A method for assaying for a ligand that is capable of disrupting the binding of a viral transforming protein to RB, while not disrupting the binding of RBAP-1 to RB comprising the steps of,
 - transforming a cell with vectors containing a reporter gene having an activatable promoter, and containing DNA encoding RB and RBAP-1 where one of the potential binding partners is fused to a transactivating domain and the other is fused to a site specific DNA binding domain,
 - transforming a cell with vectors containing a reporter gene having an activatable promoter, and containing DNA encoding RB and one viral transforming protein where one of the potential binding partners is fused to a transactivating domain and the other is fused to a site specific DNA binding domain,
 - culturing said transformed cells in the presence of said ligand,
 - determining expression of said reporter gene.

34. A method for assaying for a ligand that can disrupt the binding of a viral transforming protein to RB, while not disrupting the binding of RBAP-1 to RB comprising the steps of,

transforming a mammalian cell expressing a viral transforming protein with vectors containing a reporter gene having an activatable promoter, and containing DNA encoding RB and RBAP-1 where one of the potential binding partners is fused to a transactivating domain and the other is fused to a site specific DNA binding domain,

culturing said transformed cell in the presence of said ligand,
determining expression of said reporter gene.

35. A monoclonal antibody that is directed to the gene product of any portion of said DNA molecule in claim 23.

36. The monoclonal antibody of claim 35 comprising a monoclonal antibody directed against the peptide encoded by nucleotides 1191-1655 of Fig. 1.

1 GGGATCGAGCCCTGCCGAGGCCCTGCCGCCATGGGCCCGGCCGCCGCCGCCCTGTCA
 1
 CCCGGGCCGCCGCCGGCCGTGAGCGTCATGGCCTTGGCCGGGCCCTGCAGGGCGGCCAT
 M A L A G A P A G G P
 121 GCGCGCCGGCGCTGGAGGCCCTGCTCGGGGCCGGCGCTGCGCTGCTCGACTCCTCGC
 12 C A P A L E A L L G A G A L R L L D S S
 AGATCGTCATCATCTCCGCCGCCAGGACGCCAGCGCCCGCCGGCTCCACCGGCCCG
 Q I V I I S A A Q D A S A P P A P T G P
 241 CGGCGCCCGCCGCCGGCCCTGCGACCCCTGACCTGCTGCTCTCGCCACACCGCAGGCC
 52 A A P A A G P C D P D L L L F A T P Q A
 CCCGGCCCACACCCAGTGCGCCGCCGCGCTCGGCCGCCGGTGAAGCGGAGGC
 P R P T P S A P R P A L G R P P V K R R
 361 TGGACCTGAAACTGACCATCAGTACCTGGCCAGAGAGCAGTGGCCAGCTGGGGCAGAG
 92: L D L E T D H Q Y L A E S S G P A R G R
 GCCGCCATCCAGGAAAAGGTGTGAAATCCCCGGGGAGAAGTCACGCTATGAGACCTCAC
 G R H P G K G V K S P G E K S R Y E T S
 481 TGAATCTGACCACCAAGCGCTTCTGGAGCTGCTGAGCCACTCGGCTGACGGTGTGTCG
 132 L N L T T K R F L E L L S H S A D G V V
 ACCTGAACTGGGCTGCCGAGGTGCTGAAGGTGCAGAAGCGGCCATCTATGACATCACCA
 D L N W A A E V L K V Q K R R I Y D I T
 601 ACGTCCTTGAGGGCATCCAGCTATTGCCAAGAAGTCCAAGAACACATCCAGTGGCTGG
 172 N V L E G I Q L I A K K S K N H I Q W L
 GCAGCCACACCAAGTGGCGTCGGCGGACGGCTTGAGGGTTGACCCAGGACCTCCGAC
 G S H T T V G V G G R L E G L T Q D L R
 721 AGCTGCAGGAGAGCGAGCAGCAGCTGGACCCACCTGATGAATATCTGTACTACGCAGCTGC
 212 Q L Q E S E Q Q L D H L M N I C T T Q L
 GCCTGCTCTCCGAGGACACTGACAGCCAGCGCCTGGCCTACGTGACGTGTAGGACCTTC
 R L L S E D T D S Q R L A Y V T C Q D L

FIG. 1a

841 GTAGCATTGCAGACCCCTGCAGAGCAGATGGTTATGGTATCAAAGCCCTCCTGAGACCC
 252 R S I A D P A E Q M V M V I K A P P E T
 AGCTCCAAGCCGTGGACTCTTCGGAGAACCTTCAGATCTCCCTTAAGAGCAAACAAGGCC
 Q L Q A V D S S E N F Q I S L K S K Q G
 961 CGATCGATGTTTCTGTGCCCTGAGGAGACCGTAGGTGGGATCAGCCCTGGGAAGACCC
 292 P I D V F L C P E E T V G G I S P G K T
 CATCCCAGGAGGTCACTTCTGAGGAGGAGAACAGGGCCACTGACTCTGCCACCATAGTGT
 P S Q E V T S E E E N R A T D S A T I V
 1081 CACCACCAACCATCATCTCCCCCTCATCCCTCACACAGATCCCAGCCAGTCTACTCA
 332 S P P P S S P P S S L T T D P S Q S L L
 GCCTGGAGCAAGAACCGCTGTTGTCCCGATGGGAGCCTGCAGGGCTCCGTGGACGAGG
 S L E Q E P L L S R M G S L R A P V D E
 1201 ACCGCCTGTCCCCGCTGGTGGCGGCCGACTCGCTCCTGGAGCATGTGCGGGAGGACTTCT
 372 D R L S P L V A A D S L L E H V R E D F
 CCGGCCTCCTCCCTGAGGAGTTCATCAGCCTTCCCCACCCACGAGGCCCTGACTACC
 S G L L P E E F I S L S P P H E A L D Y
 1321 ACTTCGGCCTCGAGGAGGGCGAGGGCATCAGAGACCTTCTGACTGTGACTTTGGGGACC
 412 H F G L E E G E G I R D L F D C D F G D
 TCACCCCCCTGGATTTCTGACAGGGCTGGAGGGACCAGGGTTCCAGAGATGCTCACCT
 L T P L D F a
 1441 TGTCTCTGCAGCCCTGGAGCCCCCTGTCCCTGGCCGTCCAGCCTGTTGGAAACAT

 TTAATTATACCCCTCTCCTCTGTCTCCAGAAGCTTAGCTCTGGGGTCTGGCTACCGC

 1561 TAGGAGGCTGAGCAAGCCAGGAAGGAAGGAGTCTGTGTGGTGTATGTGCATGCAGCC

 TACACCCACACGTGTGTACCGGGGGTGAATGTGTGAGCATGTGTGTGCATGTACCG

FIG. 1b

1681 GGGATGAAGGTGAAACATACACCTCTGTGTGCAGACACGCCAGTGTGCTCA
.....
CATGTGTGTGCATGAGTCCATGTGTGCGCGTGGGGGGCTCTAACTGCACTTCGGCCCT
.....
1801 TTTGCTCTGGGGTCCACAAGGCCAGGGCAGTGCTGCTCCAGAATCTGGTGTCTGA
.....
CCAGGCCAGGTGGGAGGCCTTGGCTGGCTGGCGTGTAGGACGGTGAGAGCACTCTGT
.....
1921 CTTAAAGGTTTTCTGATTGAAGCTTAATGGAGCGTTATTATTTATCGAGGCCTCTT
.....
TGGTGAGCCTGGGAATCAGCAAAGGGAGGGAGGGTGTGGGTTGATACCCAACTCCC
.....
2041 TCTACCCCTTGAGCAAGGCAGGGTCCCTGAGCTTTCTGCCCTACTGAAGGAAC
.....
TGAGGCCTGGGTGATTTATTATTGGAAAGTGAGGGAGGGAGACAGACTGACTGACAGC
.....
2161 CATGGGTGGTCAGATGGTGGGTGGGCCCTCCAGGGGCCAGTCAGGGCCAGCTG
.....
CCCCCAGGATGGATATGAGATGGAGAGGTGAGTGGGGACCTTCACTGATGTGGCAG
.....
2281 GAGGGGTGGTGAAGGCCTCCCCAGCCCAGACCTGTGGTCCCTCTGCAGTGTCTGAAG
.....
CGCCTGCCTCCCCACTGCTCTGCCCAACCTCCAATCTGCACTTGATTTGCTTCCTAAC
.....
2401 AGCTCTGTTCCCTCCTGTTGGTTAATAAATATTTGATGACGTTAAAAAAA

FIG.1c

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl. 5 C12N15/12; C07K13/00; C12N15/62; C12P21/08 C12Q1/68; G01N33/68																			
II. FIELDS SEARCHED <table border="1"> <tr> <td colspan="4" style="text-align: center;">Minimum Documentation Searched⁷</td> </tr> <tr> <td>Classification System</td> <td colspan="3">Classification Symbols</td> </tr> <tr> <td>Int.Cl. 5</td> <td>C12N</td> <td>C07K</td> <td>C12Q</td> </tr> <tr> <td></td> <td></td> <td></td> <td>G01N</td> </tr> </table>				Minimum Documentation Searched ⁷				Classification System	Classification Symbols			Int.Cl. 5	C12N	C07K	C12Q				G01N
Minimum Documentation Searched ⁷																			
Classification System	Classification Symbols																		
Int.Cl. 5	C12N	C07K	C12Q																
			G01N																
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸																			
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹																			
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³																	
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 90, no. 8, 15 April 1993, WASHINGTON US pages 3525 - 3529 HUBER HE;EDWARDS G;GOODHART PJ;PATRICK DR;HUANG PS;IVEY-HOYLE M;BARNETT SF;OLIFF A;HEIMBROOK DC; 'Transcription factor E2F binds DNA as a heterodimer.' see page 3525, column 2, line 47 - page 3526, column 1, line 14 see page 3527, column 1, line 20 - page 3529, column 2, line 6 --- -/-	1-20, 23-31, 35,36																	
⁶ Special categories of cited documents : ¹⁰ ⁷ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art Z document member of the same patent family																			
IV. CERTIFICATION																			
Date of the Actual Completion of the International Search 04 OCTOBER 1993		Date of Mailing of this International Search Report 14-10-1993																	
International Searching Authority EUROPEAN PATENT OFFICE		Signature of Authorized Officer S.A. NAUCHE																	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	<p>MOLECULAR AND CELLULAR BIOLOGY vol. 12, no. 12, December 1992, WASHINGTON US pages 5620 - 5631 SHAN, B. ET AL.; 'Molecular cloning of cellular genes encoding retinoblastoma-associated proteins: identification of a gene with properties of the transcription factor E2F.' see the whole document ---</p>	1-36
P,X	<p>CELL vol. 70, no. 2, 24 July 1992, CAMBRIDGE, NA US pages 351 - 364 KAELIN WG JR;KREK W;SELLERS WR;DECAPRIO JA;AJCHENBAUM F;FUCHS CS;CHITTENDEN T;LI Y;FARNHAM PJ;BLANAR MA;ET AL; 'Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties.' see the whole document ---</p>	1-36
P,X	<p>CELL vol. 70, no. 2, 24 July 1992, CAMBRIDGE, NA US pages 337 - 350 HELIN K;LEES JA;VIDAL M;DYSON N;HARLOW E;FATTAEY A; 'A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F.' see the whole document ---</p>	1-36
X	<p>WO,A,8 906 703 (DRYJA,T ET AL.; US) 27 July 1989 See the abstract, claims 1-5 ---</p>	23,25-28
A		31-34
X	<p>EMBO JOURNAL: vol. 6, no. 7, 1987, EYNSHAM, OXFORD GB pages 2061 - 2068 YEE, A.S. ET AL.; 'Promoter interaction of the E1A-inducible factor E2F and its potential role in the formation of a multi-component complex' see page 2067, column 2, line 15 - page 2067, column 2, line 39 ---</p>	24-28
		-/-